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13. ABSTRACT (Maximum 200 Words) Examination of the enzyme structure for acetylcholinesterase (AChE) reveals two sites of ligand interaction: The <i>peripheral site</i> (P-site) located at the entrance of the gorge, and the <i>acylation site</i> (A-site) at the base of the gorge. Our goal is to develop high affinity cyclic peptide ligands specific for the P-site that would block the access of organophosphate agents while allowing the passage of acetylcholine to the A-site for use by personnel at risk for nerve gas exposure. Our immediate strategy involves the covalent tethering of cyclic inhibitors via a methanethiosulfonate (MTS) linkage to a cysteine on the AChE mutant, H287C. We are using a combinatorial approach to identify tethered cyclic peptides with high affinity for the P-site. The modified AChEs linked to candidate peptides that inhibit P-site access are selected by affinity chromatography. We are developing mass spectrometry techniques to determine the peptide structure of these candidates. These include release of the cyclic peptide by reduction with DTT and peptide sequencing by 1 dimensional liquid chromatographic ESI ion-trap mass spectrometry. This method should provide the peptide amino acid sequence through its MS ⁿ capabilities, which allow for peptide fragment analysis through several stages of consecutive collisionally activated decomposition (CAD) mass spectra.				
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INTRODUCTION

Our studies focus on the enzyme, acetylcholinesterase (AChE), which is responsible for hydrolyzing the neurotransmitter, acetylcholine (ACh). AChE is a key enzyme at the neuromuscular junction with one of the fastest known enzymatic rates. (1) X-ray crystallography of this enzyme reveals a large active site gorge made up of two distinct sites separated by a 20Å narrow channel. At the base of this gorge is the acylation site (A-site), while close to the mouth of the gorge is the peripheral site (P-site) (2). The presence of two enzymatic sites allows for binding of ligands to either the A-site or P-site as well as to both sites simultaneously. AChE is inactivated by organophosphates (OPs) in pesticides and chemical warfare agents because OPs can pass through the P-site and phosphorylate the catalytic serine in the A-site. OP inactivation of AChE results in failure of cholinergic synaptic transmission, deterioration of neuromuscular junctions, flaccid muscle paralysis, and seizures in the central nervous system.

Our strategy has been to focus on the P-site to design inhibitors that would selectively inhibit OP inactivation of AChE while still allowing for the passage of ACh to the A-site. These inhibitors would act as prophylactics to protect against attack by nerve agents while still providing for the normal hydrolysis of ACh by AChE. In recent years we have found that ligands that bind to the P-site inhibit AChE through a process we call *steric blockade* (3,4). This process involves a decrease in the rate constants with which substrates and their hydrolysis products enter and exit the A-site. The concept of steric blockade has led us to a new strategy for the design of drugs to protect AChE from inactivation by OPs. Such a drug must exclude OPs from the acylation site while interfering minimally with acetylcholine passage. Our search for a prototype of this drug has focused on cyclic peptide and pseudopeptide compounds, as they have a number of advantages. First, a cyclic molecule is a ring with a pore that in theory can be designed to exclude passage of bulky OPs while allowing smaller acetylcholine to pass. Relatively small cyclic compounds consisting of 6 to 8 amino acids possess the necessary space to permit passage of acetylcholine. Second, the incorporation of both natural and unnatural amino acids using combinatorial methods allows for synthesis of an enormous number of cyclic compounds in libraries of various size. Furthermore, cyclic peptides are conformationally constrained, an asset in molecular modeling studies.

Initial experiments showed that 7- and 8-residue cyclic peptides with two or three cationic groups had detectable affinity for the P-site at 100 µM concentrations (5). The next series of cyclic compounds was modeled on the snake neurotoxin fasciculin (Fas), which is specific for the P-site and displays an impressive affinity for the enzyme ($K_D \sim 10 - 20$ pM) (6,7). Fas consists of three finger-like loops protruding from a central disulfide linked core, and crystal structures of the AChE-Fas complex (8,9) show that loop II displays the greatest area of contact with the AChE P-site. The loop II primary sequence -Arg-Arg-His-Pro-Pro-Lys-Met- was cyclized by insertion of a Gln residue, and substitutions at Arg² and Lys⁶ (which do not make direct contact with the enzyme surface) were investigated. The cyclic peptide cyclo[Arg-Nal-His-Pro-Pro-Lys-Met-Gln], where Nal is naphthylalanine, had the highest affinity and was found to inhibit AChE with an inhibition constant K_i of less than 1 µM (5). However, we were concerned about too great an emphasis on this Fas-based cyclic peptide. The crystal structures showed that insertion of Fas loop II into the AChE P-site results in contacts of both faces of loop II with AChE residues. These contacts are extensive, and surface calculations showed that not even a water molecule would be able to enter the active site in the crystal structure of the complex (9). Therefore, high affinity cyclic peptides based on Fas loop II may be misleading

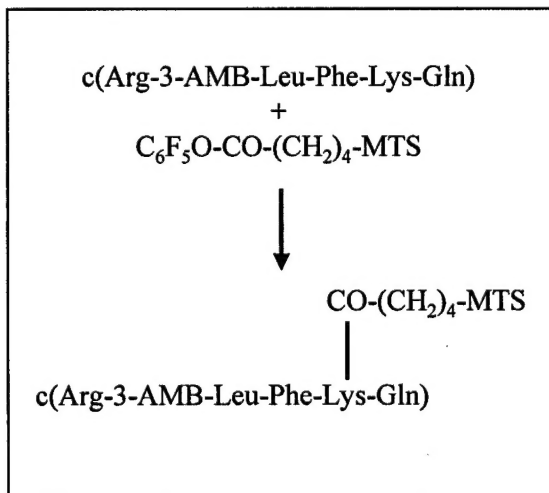
structural prototypes if our goal is to build a high affinity pore at the peripheral site that still allows access of acetylcholine.

If we move beyond Fas loop II as a model, it becomes a challenge to identify a cyclic peptide with sufficient affinity for the AChE P-site to serve as a lead compound for further development. To confine candidate peptide interactions to this site, we have developed a procedure for covalently tethering cyclic peptides near the P-site. Tethering is an intermediate strategy, and we anticipate that high affinity inhibitors identified with this approach will retain their high affinity when the tether is removed. The site selected for tethering was H287, a residue on the rim of the P-site but with a side chain that extends outward into solution. We have developed a protocol for tethering prospective compounds to the P-site by preparing a mutant form of the human AChE that substitutes a cysteine for the histidine at residue 287, designated as H287C AChE. Our candidate cyclic peptides have been designed to include a Lys residue, and we attach a linker to the ϵ -amino group of this Lys. The linker terminates with a methanethiosulfonate group (MTS), and reaction of this substituent with the -SH group of residue C287 creates a covalent disulfide bond that localizes the cyclic peptide near the P-site. After attachment of the cyclic peptide to the enzyme we can evaluate binding to the enzyme by affinity chromatography on acridinium resin. In our progress report for this grant last year, we demonstrated proof of concept for this approach with a series of tethered cationic ligands that were linked to C287. Unmodified AChE was retained on the acridinium resin and eluted only under stringent conditions with the AChE inhibitor decamethonium. Tethered ligands that reached the P- and or A-sites interfered with binding of the modified AChE to the acridinium resin and were eluted from the resin by a less stringent salt wash (10). The location of the tethered ligands were predicted by molecular modeling calculations and verified experimentally by testing the modified enzymes for inhibition with propidium (a P-site inhibitor) and tacrine (an A-site inhibitor). We demonstrated that tethered ligands that reach only the P-site selectively blocked propidium inhibition, while tacrine affinity for the A-site decreased when tethered ligands reach the A-site (10).

BODY

Our preliminary data indicate that the design of cyclic peptides with high affinity for the P-site will be a multi-step process. The tethering strategy provides an important first step by limiting the interaction of a candidate peptide to the immediate vicinity of the P-site. A second step is to develop a combinatorial approach that allows high affinity peptides to be selected from peptide mixtures so that larger numbers of peptides can be screened. Ligand-based affinity chromatography looks very promising as a selection tool, as the tethering of high affinity peptides will prevent strong retention of modified AChEs. We are developing cyclic peptide synthesis procedures that will provide appropriate peptide mixtures. We require purification procedures that retain all peptides in the mixture but remove synthesis byproducts that interfere with either the attachment of tethers to the peptides or the reaction of the tethered peptides with AChE. In parallel with this second step, we are seeking molecular modeling programs that can assist in cyclic peptide design by predicting relative affinities of tethered peptides to the P-site. This has been problematic, as the majority of both commercially and academically available molecular docking software applications we have explored so far focus on elucidating the binding of small, rigid molecules to a cavity removed from aqueous solvent. We require a program that will assist in designing large, flexible tethered cyclic peptides that bind tightly to the P-site of AChE with one face while the other face is left exposed to solvent. While seeking such a program, we are making some progress by adapting our queries to match the capabilities of the software. In programs such as InsightII (Accelrys, Inc.), we have broken up the targeted rim of the P-site into smaller pieces and probed these areas with small, rigid ligands. This approach suggested, for example, that Fas-based cyclic peptides were inserting themselves into the P-site rather than binding at the rim as intended. A third step is to employ mass spectrometry to identify tethered peptides that are selected by affinity chromatography. Tethered peptides can be tentatively identified by their total mass, although peptides with identical masses would not be resolved by mass alone. Alternatively, mass spectrometry that allows analysis of both parent peptide ions and their collision-induced fragments (MS/MS) can permit peptide sequencing and unequivocal peptide identification. We have explored both of these approaches as outlined below.

Cyclic peptide synthesis. We have advanced our synthesis strategy to include the attachment of the MTS-linker group to a primary amino group (e.g., Lys) of the candidate cyclic peptide. The starting cyclic peptides were obtained by solid phase peptide synthesis (11) using an Fmoc- synthesis strategy on MBHA resin, followed by on-resin cyclization and final deprotection and cleavage from the resin by liquid anhydrous HF. Conversion of the cyclic peptides to their MTS-derivatives was conducted according to the adjacent scheme (with c(Arg-3-AMB-Leu-Phe-Lys-Gln) as an example, where 3-AMB is 3-aminomethylbenzoic acid).



Purification by solid phase extraction gave a 37 – 70% yield (fraction scanned by analytical HPLC and MALDI-TOF MS). A solution-phase approach was used also to attach the tether 8-amino-3,6-dioxaoctanoic acid (mini PEG) in amide linkage to the amino group of the Lys side chain of a cyclic peptide, and the MTS-propionyl residue then was inserted at the end of this linker.

[3H]H287C-labeling and Affinity Chromatography. Our strategy for attaching MTS-linker-peptides to H287C AChE and screening for blockade of the P-site was developed with a series of MTS-derivatized cationic ligands (10) and presented in detail in our progress report for this grant last year. In brief, we first labeled the amine groups in the AChE by reductive methylation with [³H]formaldehyde and sodium cyanoborohydride (12). This procedure has no effect on the enzyme activity of unmodified AChE, and it allows precise quantitation of the enzyme after modification with MTS compounds that alter the enzyme activity. Radiolabeled H287C AChE was reduced with dithiothreitol (DTT), dialyzed, and reacted with 2 mM MTS-linker-peptide for 30 min at room temperature (10). The sample was dialyzed to remove excess unreacted or hydrolyzed MTS compound and applied to an acridinium resin affinity column (5 ml). The column was washed sequentially with 10 mM sodium phosphate buffer, 10 mM buffer containing 0.5 M NaCl (salt wash), and 5 mM buffer containing 0.5 M NaCl and 5 mM decamethonium bromide, a specific AChE inhibitor (deca wash) (10). Collected fractions were monitored for enzyme activity with the Ellman assay and for protein content by liquid scintillation counting. Enzyme eluted in the salt wash represents modified AChE that has decreased affinity for the acridinium resin and shows weaker inhibition by an A-site inhibitor (tacrine) and a P-site inhibitor (propidium) (10). This protocol has been applied to H287C AChE and 15 MTS-linked cyclic peptides (Table 1 and Table 2) since our last report. In Table 1 we focus on a group of compounds based on the cyclic peptide c(Arg-3-AMB-Leu-Phe-X-Gln), where X was either Lys, D-Lys or DAP. The length of hydrophobic 3-AMB corresponds to about 1½ peptide bond lengths. This cyclic peptide was selected because it showed some affinity for the P-site as a reversible AChE inhibitor. Changes in the X residue were made to examine the length of the side chain attached to the linker. In addition, we looked at insertion of miniPEG (-NH-(C₂H₅O)₂-CO-) in the linker to increase solubility and hydrophilicity. The results in general indicated that we need to survey additional cyclic peptide structures. AChE modification with only 2 of the compounds in Table I (IV and VII) resulted in any enzyme release from the affinity resin in the salt wash, with release in other cases requiring decamethonium. One early compound (XV) based on the Fas structure could not be analyzed because of solubility problems, but we will return to this class of peptides now that we have linkers with better hydrophilicity. Even though we have reservations about Fas-based peptides as noted in the Introduction, they should at least provide better affinity for the P-site. While we will continue to explore individual MTS-linker-peptides, particularly those that incorporate segments from Fas loop 2, we will increasingly investigate mixtures prepared in combinatorial syntheses to accelerate identification of tethered cyclic peptides with high affinity for the P-site.

Confirmation of Cyclic Peptide Tethering by Analysis of the Kinetics of Fasciculin Binding. One concern when we fail to see any change in activity or acridinium resin affinity of an AChE after conducting an MTS-linker-peptide modification reaction is whether a significant fraction of the AChE was actually modified. One approach is to change the AChE radiomethylation label to ¹⁴C and introduce a ³H-radiolabel into the MTS-linker-peptide so that

reaction stoichiometries can be determined by dual isotope scintillation counting. We are currently exploring synthesis strategies to incorporate ^3H into the cyclic peptide or linker synthesis. In addition, we will employ a kinetic assay that should be very sensitive to any modification near residue 287. We have previously demonstrated that Fas can be used to monitor interactions at the P-site (13). We now propose to use changes in the k_{on} rate for Fas to confirm the presence of compounds tethered at the H287C site. To illustrate the procedure, acetylthiocholine (AcTCh) hydrolysis was monitored by continuous spectrophotometric assay in 1 ml assay solutions with buffer (20mM sodium phosphate and 0.02% Triton X-100 at pH 7.0 at 25 °C). Assay solutions included 0.6 mM AcTCh and 0.33 mM DTNB, and hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm. Association reactions (1 ml) were initiated by adding small volumes of Fas (1 nM) to AChE (10 pM), followed by the immediate addition of DTNB and AcTCh. Assay rates v over 2 s intervals were fitted by nonlinear regression analysis to eq 1. In eq 1, v_{initial} and v_{final} are the calculated values of v at time zero and at the final steady state when Fas binding has reached equilibrium, and k is the observed rate constant for the approach to equilibrium.

$$\text{Equation 1} \quad v = v_{\text{final}} = (v_{\text{initial}} - v_{\text{final}})e^{-kt}$$

The observed rate constant k for each reaction is determined by k_{on} and k_{off} as given in eq 2, where k_{on} is the apparent association rate constant and k_{off} is the apparent association rate constant.

$$\text{Equation 2} \quad k = k_{\text{on}}[\text{Fas}] + k_{\text{off}}$$

In Figure 1 we demonstrate measurements of k for wild type (wt) AChE, H287C AChE, and H287C modified with MTS-linker peptide I (Table 1). The k values are essentially the same for wt AChE and unmodified H287C. The enzyme treated with compound I eluted from acridinium resin affinity only with decamethonium, indicating that any modification was not apparent by this, our primary criterion for P-site blockade. However, after extensive dialysis to remove decamethonium, this enzyme displayed a lower k for Fas association, indicating that the attached cyclic peptide did interfere with Fas binding. This assay thus can confirm the attachment of linker peptide to H287C AChE even when the peptide fails to alter acridinium resin binding or enzyme activity.

Mass Spectrometry (MS). Since one component of our strategy is to identify the tethered cyclic peptides that are selected by our affinity chromatography screening procedure, we need to distinguish those compounds in the combinatorial starting set that have blocked access to the P-site. Mass spectrometry (MS) has emerged as an effective technique for the characterization of peptides and proteins. It has proven to be an indispensable tool because it is able to provide accurate molecular mass on low picomole and even femtomole amounts of peptides. With the addition of MS/MS capabilities, sequence analysis of peptides becomes possible. Last year our Department of Neuroscience at Mayo Clinic Jacksonville purchased a ThermoFinnigan DecaXP Plus ion trap mass spectrometer with liquid chromatography interface to support three projects, one of which was this project. In addition, the department also has an older Applied Biosystems, Voyager-DERP MALDI-TOF mass spectrometer that we have found useful. Bernadette Cusack, a senior research analyst in my laboratory, has spent much of the past year obtaining training and

developing techniques in mass spectrometry, and we have gained perspective on the most effective ways to apply these techniques to our project. We review our progress in the following sections.

ESI Total Mass Measurements. The technique referred to as “top down” analysis allows direct determination of the total mass of the enzyme with its tethered cyclic peptide. These molecular mass measurements were carried out on our LCQ-DECA XP ion trap mass spectrometer (ThermoFinnigan, San Jose, CA).

Samples including the controls horse myoglobin (16952 Da), bovine serum albumin (BSA) (66430 Da) and affinity purified recombinant human AChE (60730 Da), were directly infused into the electrospray source fitted with a 30 μm metal needle at a flow rate of 3 $\mu\text{L}/\text{min}$ using the continuous infusion Hamilton syringe pump accessory. Compounds were dissolved in methanol:H₂O:acetic acid 60:40:0.1% at 400 ng/ μL , and mass spectra were acquired by scanning a m/z range from 700-2000. Spray voltage was set at 4.5 kV, capillary temperature was set to 220 °C, capillary voltage was 132 kV, and tube lens was 198. Mass spectra were averaged over the total time of collection (1-5 min) before performing deconvolution using the Biomass program included in the Xcalibur software.

In Figure 2a, the envelop of charged peaks, representing various charged states z of myoglobin, was deconvoluted to give an observed mass of 16956.8 (inset). Figure 2b is the analysis of BSA with a deconvoluted mass of 66933.4 (inset). However, we could not resolve discrete multiply charged species for either wt AChE or H287C AChE. In an attempt to improve the resolution of charged peaks for both BSA and wt AChE we reduced and alkylated both compounds (800 μg). Reduction was completed with DTT [5 mM] for 1 hour at room temperature, followed by treatment with iodoacetamide [40 mM] for 1 hour at room temperature in the dark. Samples were taken to dryness on a Speed-Vac and stored at -20 °C until ready for MS analysis. Figure 2c is the result obtained for the reduced and alkylated BSA. Rather than improve resolution, the envelop was more poorly resolved into discrete peaks. For the reduced and alkylated wt AChE (data not shown) there were no discrete peaks and no evidence of an envelop. Others have reported similar results with wt AChE and were only able to obtain a molecular mass after expressing the monomeric form (C580S AChE) in a bacterial system where the enzyme is not glycosylated (14).

ESI Tryptic Peptide Fragmentation and Identification. We also explored the technique referred to as the “bottom up” proteomics approach to identify the tryptic peptide fragment of a modified AChE that is attached to the tethered cyclic peptide.

After reduction and alkylation as above, modified AChEs were dialyzed against 50 mM NH₄HCO₃ and denatured by addition of acetonitrile (CH₃CN) to a final concentration of 30% for 15 minutes at room temperature. Excess CH₃CN was removed on a Speed-Vac vacuum dryer until a final concentration of 5% was obtained (15), and samples were digested overnight at 37 °C after addition of trypsin (Sigma, proteomics grade) (1:20 w/w trypsin:protein). Samples were dried, dissolved in CH₃CN:0.1% formic acid (loading solvent) to a final concentration of 5 pmol/10 μL , and filtered (0.2 μm filter) for introduction to the nanospray source of the LCQ-DECA XP. Samples (60 fmol – 5 pmol) were automatically injected onto a reversed-phase

PicoFit column (75 μ m ID x 10cm, 5 μ m BioBasic C18 from New Objective, Inc). The flow rate through the picofrit was adjusted to 200 nl/min, and the electrospray source parameters were 1.7 kV of applied voltage with capillary temperature at 170 °C, capillary voltage at 36 V, and tube lens offset at 22 V. Tryptic peptides were separated using a gradient from 99% A:1% B to 20% A:80% B over 45 minutes, where mobile phase A was aqueous formic acid (0.1% in H₂O) and B was CH₃CN + 0.1% formic acid. A full MS scan from m/z 200-2000 was first acquired for the eluting tryptic peptides, followed by a MS/MS scan between m/z 200-2000 of the most intense ion in the previous full MS scan. Once sampled, each MS/MS precursor mass was excluded from further tandem experiments for 3 minutes. For each full MS scan, the 3 most intense peaks were selected for MS/MS analysis. Acquired MS/MS spectra were searched against a human protein database (FASTA) using the SEQUEST algorithm (ThermoFinnigan, San Jose, CA). The sequences of peptides identified through SEQUEST were verified by manual examination of the corresponding MS/MS spectrum, and additional spectra for tryptic fragment masses predicted from the AChE sequence were also manually examined to assure complete coverage of all possible peptide fragments.

The Pepcut program on the Sequest browser provided predicted masses of all AChE tryptic peptides (37 peptides of 3 or more residues). Peptide 275-296 was of the greatest interest because it contained the cysteine with the tethering site in H287C AChE (Table 3). While we were able to identify several distinct tryptic peptides from digests of wt and H287C AChE, it was unfortunate that we did not find peptide 275-296 in either digest. To determine the elution time for this peptide and to provide an internal standard, we had the corresponding stable isotopic peptide synthesized (Mayo Proteomics Research Center, Rochester, MN) with Val²⁹⁴ containing 5 x ¹³C and 1 x ¹⁵N atoms for an increase of 6 average mass units (amu) (Table 3). Following direct infusion of this 22mer on the LCQ-DECA XP, MS/MS analysis on m/z 1308 (the +2 charge state) gave a fragment ion profile with several predicted b and y ions sufficient to give clear peptide identification (Figure 3; Inset is full MS scan). In the full MS scan the 1309 peak represents the +2 charge state while 873.3 peak is the +3 charge state. We then ran a 500 fmol sample of this 22mer through the LC and nanospray source, identified the +2 and +3 charge states of the 22mer, and successfully confirmed the sequence of the peptide from MS/MS scans (data not shown). Returning to the wt AChE tryptic digest, we added 500 fmol of the 22mer to 1-5 pmoles of digest and repeated the LC and nanospray MS analysis. Examination of the resulting spectra with either the SEQUEST software or manually failed to resolve peaks with the predicted masses of either peptide 275-296 or the added 22mer. The basis for the suppression of these peaks was unclear.

MALDI TOF Identification of Tethered Peptides Released by Reduction. Another MS strategy for identifying the tethered cyclic peptide in a modified AChE was to reduce the disulfide linkage with DTT and then identify the released tethered peptide by MS. Initial experiments were conducted with MTS-linker-peptide II (see Table 1), unmodified H287C, and H287C modified with compound II. Compound II (mass = 1117) loses the MTS group during reduction (-79.1 amu) to yield a mass of 1037.9. MS measurements were made with a Voyager-DERP MALDI-TOF (Applied Biosystems, Foster City, CA) in the linear mode with external calibration. Samples (20 pmol) were dissolved in a solution of the α -cyano-4-hydroxycinnamic acid (CHCA) matrix. The MS scan of compound II alone in solution showed a major peak of 1117.75 corresponding to the intact compound and a peak at 1039.07 representing the compound

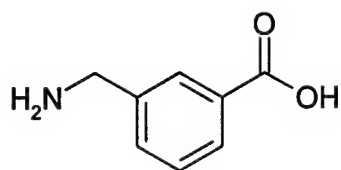
minus MTS. Also present was a peak at 2078.65, probably a disulfide-linked dimer of compound II without an MTS group. The scan for the reduced unmodified H287C (Figure 4b) showed no peaks in the 500-3000 m/z range, as expected for this control. Reduction of H287C modified with compound II gave a major peak with a m/z of 1039.96 (Figure 4c), which corresponds closely to the reduced mass we obtained for compound II in Figure 4a. The inset in Figure 4c is a full scan of the sample from 400-2500 m/z . We concluded that release of the tethered peptide from modified H287C AChE by disulfide reduction provides a feasible way to obtain the mass of the tethered peptide.

LC ESI of Cyclic Peptides. We are interested not only in determining the mass of a tethered cyclic peptide but also in identifying it by its sequence. High-energy, collisionally activated decomposition (CAD) conducted in multiple stages on an ESI ion trap mass spectrometer is used to sequence cyclic peptides (16). The first stage involves ring opening of the cyclic peptide and is followed by several stages of CAD directed to a precursor ion and its decomposition products until an unambiguous sequence is established. We tested this method with MTS-linker-peptides II and III (Table 1), which differ by the inclusion of either Lys or DAP in the cyclic peptide. DAP is a homolog of Lys with a side chain that is three methylene groups shorter. Results with compound III are illustrated in Figure 5. The full MS scan in the inset indicates the intact compound III (mass = 1074) and the reduced compound that has lost the MTS group (mass = 997). MS/MS analysis of the 997 mass reveals that the linker is readily lost in the first CAD stage, consistent with previous observations on cyclic peptides containing linear peptide branches (16). Ring opening and loss of an NH group results in a prominent peak at m/z = 747.3, but smaller fragments also are produced that indicate loss of additional residues from these linear peptides. In this spectrum, four 3- to 5-residue fragments from three distinct linear hexapeptides (R α LF δ Q, δ QR α LF, and F δ QR α L) are assigned. We concluded from these studies that we could assign cyclic peptide structures selected from combinatorial peptide mixtures based on the combination of parent ion masses and specific sequence information from MS/MS analyses with CAD.

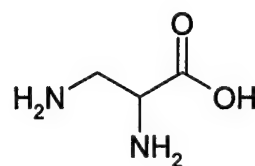
Table 1. MTS-linker-cyclic peptide compounds

ID	Linker*	Cyclic Peptide
I	MTS-propionyl-mini PEG	c(Arg-3AMB-Leu-Phe-D-Lys-Gln)
II	MTS-propionyl-mini PEG	c(Arg-3AMB-Leu-Phe-Lys-Gln)
III	MTS-propionyl-mini PEG	c(Arg-3AMB-Leu-Phe-DAP-Gln)
IV	MTS-(CH ₂) ₂ -CO-	c(Arg-3AMB-Leu-Phe-Lys-Gln)
V	MTS-(CH ₂) ₄ -CO-	c(Arg-3AMB-Leu-Phe-Lys-Gln)
VI	MTS-CH ₂ -C ₆ H ₄ -CH ₂ -CO-	c(Arg-3AMB-Leu-Phe-Lys-Gln)
VII	MTS-(CH ₂) ₂ -CO-	c(Arg-3AMB-Leu-Phe-D-Lys-Gln)
VIII	MTS-(CH ₂) ₄ -CO-	c(Arg-3AMB-Leu-Phe-D-Lys-Gln)
IX	MTS-CH ₂ -C ₆ H ₄ -CH ₂ -CO-	c(Arg-3AMB-Leu-Phe-D-Lys-Gln)
X	MTS-propionyl-	c(Arg-3AMB-Leu-Phe-Lys-Gln)
XI	MTS-propionyl-	c(Arg-3AMB-Leu-Phe-DAP-Gln)

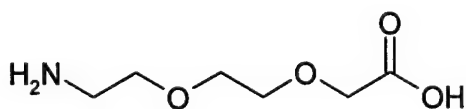
*The linker is attached either to the N-epsilon-amino group of Lys or the N-beta-amino group of diaminopropionic acid.



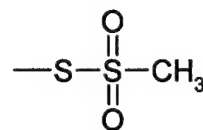
3-(aminomethyl)benzoic acid
(3-AMB)



2,3-diaminopropionic acid
(DAP)



8-amino-3,6-dioxaoctanoic acid
(mini PEG)



Methanethiosulfonyl
(MTS)

Table 2. Additional Compounds tested

ID	Linker	Cyclic Peptide
XII	MTS-(CH ₂) ₄ -CO	Phe-Arg-Lys-Arg-Lys-Arg-Ser-Arg
XIII	MTS-CH ₂ -C ₆ H ₄ -CH ₂ -CO	Phe-Arg-Lys-Arg-Lys-Arg-Ser-Arg
XIV	MTS-CH ₂ -C ₆ H ₄ -CH ₂ -CO	Gly-Phe-Arg-Lys-Arg-Lys-Arg-Ser-Arg
XV	MTS-CH ₂ -C ₆ H ₄ -CH ₂ -CO	c(Arg-Ala-His-Pro-Pro-Lys-[D-Nal]-Asn)
XVI	MTS-CH ₂ -C ₆ H ₄ -CH ₂ -CO-NH-(CH ₂) ₇	Gly-NH -(CH ₂) ₇ -HN-tetrahydroacridine
XVII	MTS-(CH ₂) ₃ -CO	NH-(CH ₂) ₇ -HN-tetrahydroacridine
XVIII	MTS-rhodamine	None
XIX	MTS-CH ₂ -C ₆ H ₄ -CH ₂ -CO-NH-(CH ₂) ₅ -CO	NH-(CH ₂) ₃ -NH-tetrahydroacridine
XX	MTS-CH ₂ -C ₆ H ₄ -CH ₂ -CO-NH-(CH ₂) ₇	HN-tetrahydroacridine
XXI	MTS-CH ₂ -C ₆ H ₄ -CH ₂ -CO-OH	None
XXII	None	c(Arg-Nal-His-Pro-Pro-Lys-Met-Gln)
XXIII	None	c(Arg-3AMB-Leu-Phe-DAP-Gln)
XXIV	None	c(Arg-3AMB-Leu-Phe-Lys-Gln)
XXV	None	H ₂ N-(CH ₂) ₇ -HN-tetrahydroacridine
XXVI	None	Tetrahydroacridine-NH-(CH ₂) ₇ -HN-tetrahydroacridine
XXVII	None	Phe-Arg-Lys-Arg-Lys-Arg-Ser-Arg-OH
XXVIII	None	c (Arg-Ala-His-Pro-Pro-Lys-Nal-Gln)
XXIX	None	c(Arg-Ala-His-Pro-Pro-Lys -[D-Nal]-Gln)
XXX	None	c(Arg-[D-Nal]- His-Pro-Pro-Lys -Met-Gln)
XXXI	None	c(Arg-Nal-His-Pro-Pro-Lys-Met-Gln)

Table 3. Comparison of Tryptic fragment 275-296 from wtAChE, H287C and 22mer

ID	Fragment 275-296 sequence	m/z charge state		
		+1	+2	+3
WtAChE	TRPAQVLVNHEWHVLPQESVFR	2642.4	1321.7	881.5
H287C	TRPAQVLVNHEWCVLPQESVFR	2608.3	1304.7	870.1
22mer	TRPAQVLVNHEWCVLPQESV*FR	2614.3	1307.7	872.1

V* = + 6 amu

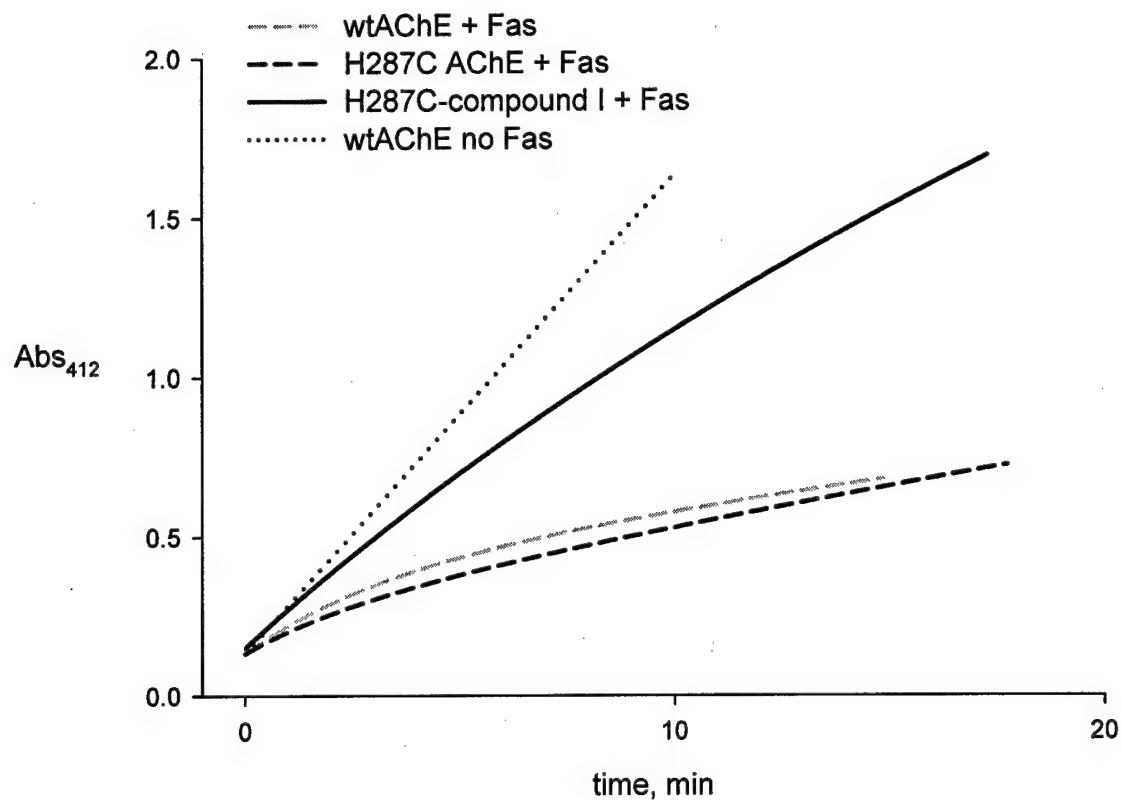


Figure 1. Fasciculin (Fas) association assay with AChE. The rate constant k for fasciculin association was measured according to equation 1 from the rate of approach to the steady state in this continuous spectrophotometric assay of acetylthiocholine hydrolysis. Samples treated with 1 nM Fas at time zero include control wt AChE, H287C AChE, and H287C AChE modified with MTS-linker-peptide I. The measured k values were 0.29 min^{-1} with wt AChE, 0.32 min^{-1} for H287C, and 0.08 min^{-1} for H287C modified with compound I. The dotted line corresponds to an assay with wt AChE in the absence of Fas.

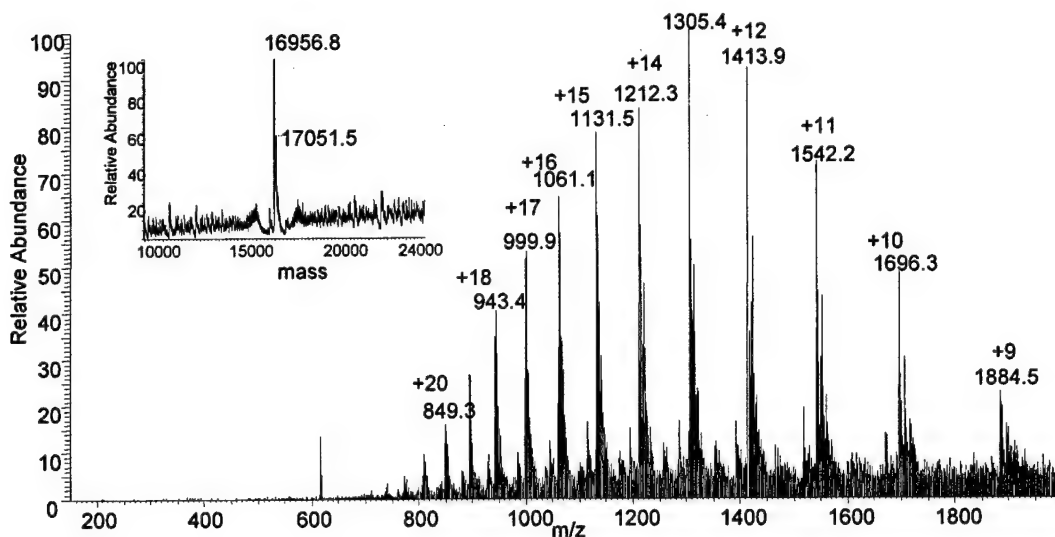


Figure 2a. Total mass determination of horse myoglobin with the ESI ion trap mass spectrometer in direct infusion. The envelop of charged peaks, representing various protonated states, has been deconvoluted using the BIOMASS program to give an observed mass of 16956.8(inset).

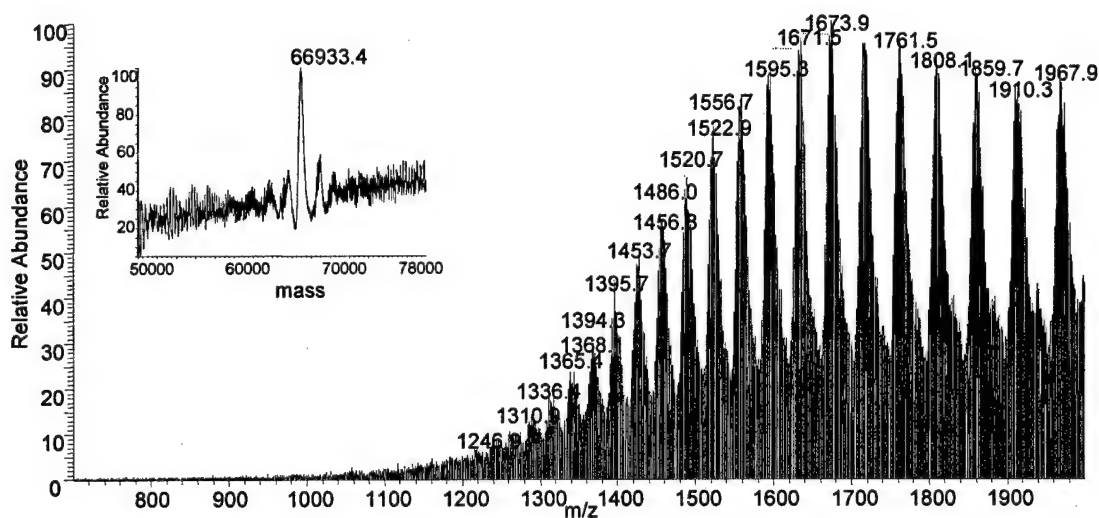


Figure 2b. Total mass determination of BSA with the ESI ion trap mass spectrometer in direct infusion mode. The deconvoluted mass (inset) of 66933.4 was obtained after optimizing scan conditions for the mass of BSA.

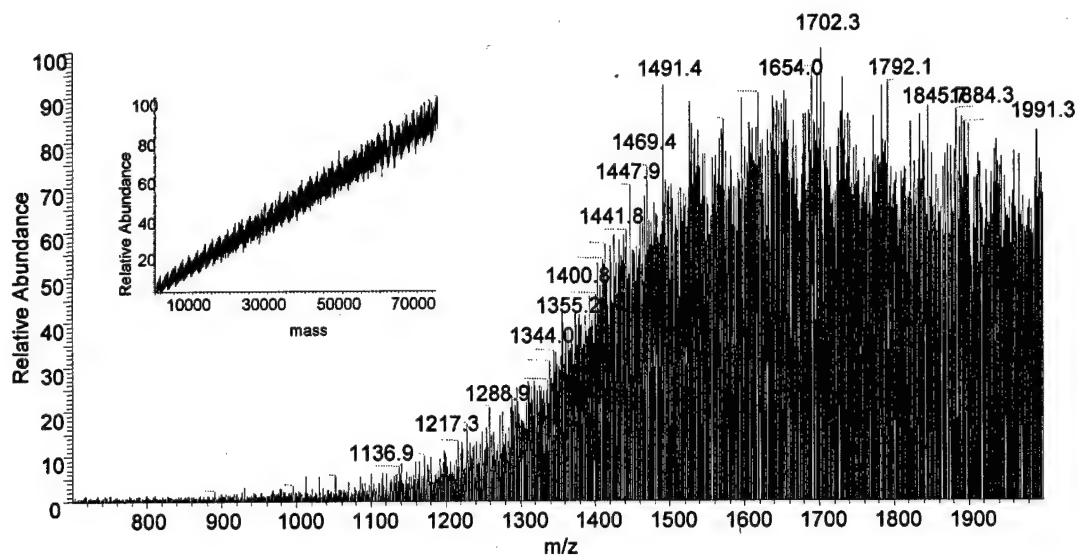


Figure 2c. Total mass determination of reduced and alkylated BSA with the ESI ion trap mass spectrometer in direct infusion mode. Discrete peaks in the envelop cannot be resolved and therefore, total mass (inset) could not be determined.

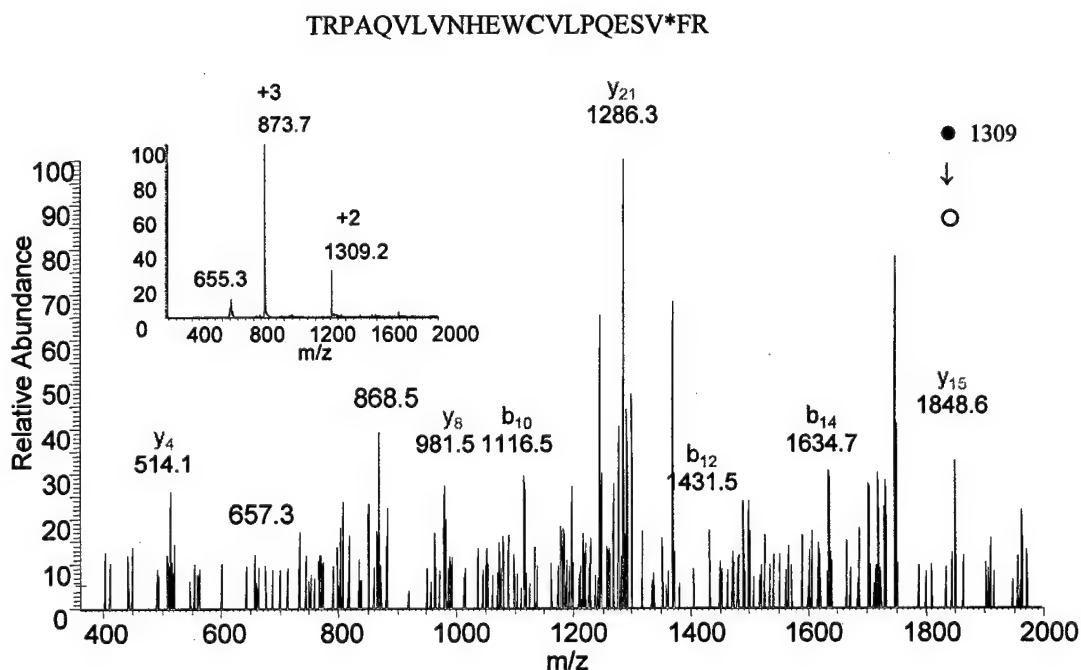


Figure 3. Full MS scan (inset) of isotopic 22mer (mass = 2614) followed by MS² on 1309 m/z peak (+2 charge state) using the ESI ion trap mass spectrometer in direct infusion mode. Fragment b and y ions are labeled and provide for sequence information for the infused 22mer.

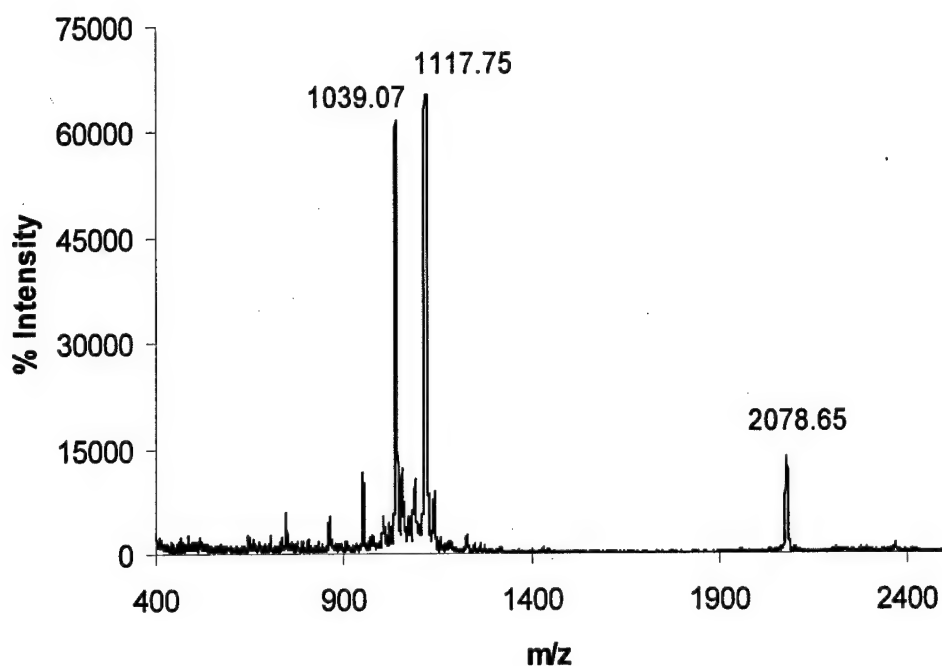


Figure 4a. MALDI-TOF scan of MTS-linker-peptide II (MTS-propionyl-mini PEG-[c(Arg-3AMB-Leu-Phe-Lys-Gln)]). Peak $m/z = 1117.75$ represents the nonreduced compound while m/z of 1039.07 is the reduced compound with the loss of MTS. The 2078.65 peak is the disulfide-linked dimer of the 1039.07 peak.

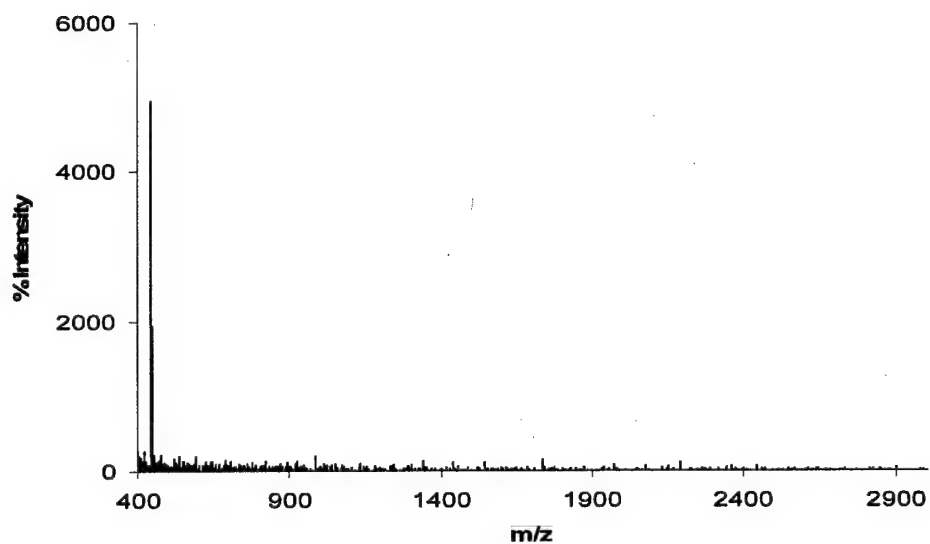


Figure 4b. MALDI-TOF scan of reduced unmodified H287C.

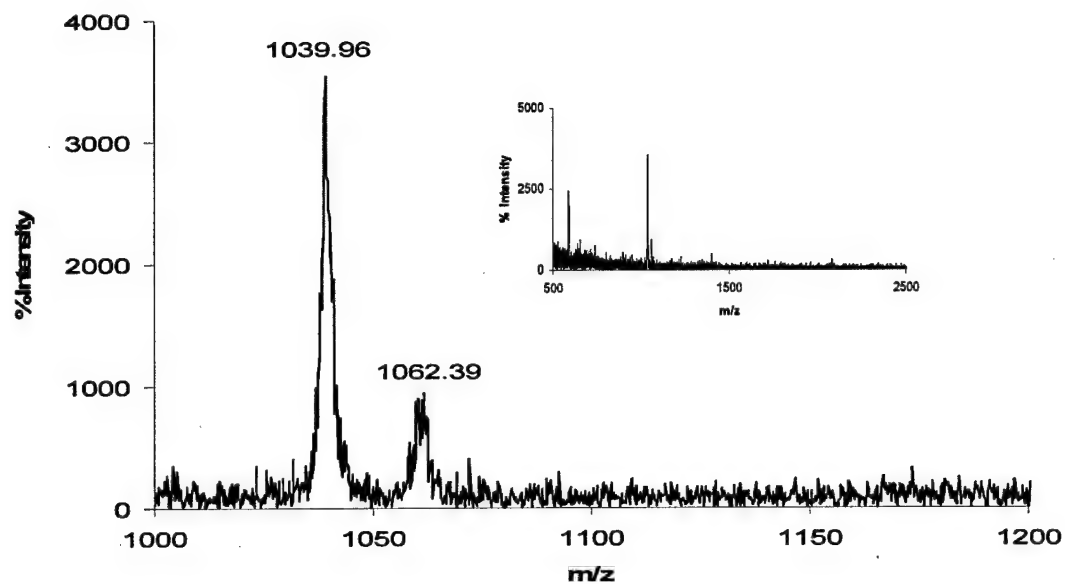


Figure 4c. MALDI-TOF scan of reduced H287C modified with compound II (inset). Zoom scan shows a peak with $m/z = 1039.96$ corresponding to the reduced compound II in Figure 4a. The minor peak at $m/z = 1062.39$ represents the sodium adduct of the major peak.

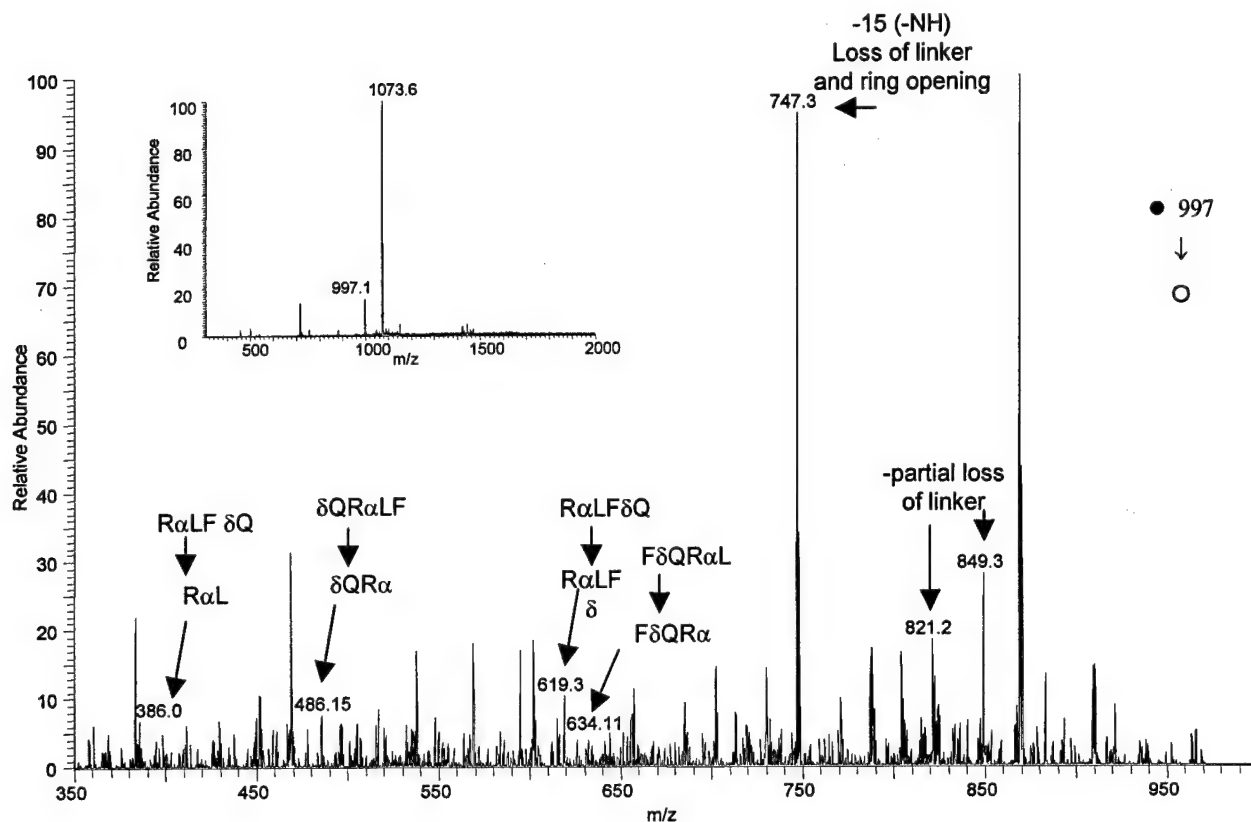


Figure 5. Full MS scan (inset) of MTS-linker-peptide III (MTS-propionyl-mini PEG-[c(Arg-3AMB-Leu-Phe-DAP-Gln)]) with the ESI ion trap mass spectrometer in direct infusion mode indicates the intact compound ($m/z = 1073.6$) and the reduced compound without the MTS group ($m/z = 998.1$). Subsequent MS/MS of the 997 ion generated selected peaks that have been labeled to indicate their significance in the sequence identification of compound III. The prominent peak at 747.3 corresponds to loss of the linker and ring opening with loss of an NH group. Additional peaks are labeled with their 6-residue parent sequence, which indicates the peptide bond broken during ring opening, and the subsequent 3- to 5-residue fragment produced ($\alpha = 3AMB$; $\delta = DAP$).

KEY RESEARCH ACCOMPLISHMENTS

- We have advanced our novel strategy of tethering MTS cyclic peptides to the H287C mutant AChE to include 15 new compounds. Several linkers have been compared and evaluated for ease of synthesis, optimum length and solubility.
- We focused on a group of compounds based on the cyclic peptide c(Arg-3AMB-Leu-Phe-X-Gln), where AMB was 3-(aminomethyl)benzoic acid and X was either Lys, D-Lys or 2,3-diaminopropionic acid. AChE modification with only 2 of the MTS-linker-peptides (IV and VII in Table I) resulted in any enzyme release from the affinity resin in the salt wash, our selection criterion for P-site blockade. The results in general indicated that we need to increase the diversity of cyclic peptide structures that we are surveying.
- We developed an assay with the neurotoxin fasciculin (Fas) to monitor attachment of MTS-linker-peptides to Cys287. A decrease in the k_{on} rate constant for Fas binding to the P-site indicated attachment of the peptide.
- We have explored the use of mass spectrometry as a powerful tool to identify candidate cyclic peptides from initial combinatorial sets. We demonstrated that we could apply MALDI TOF mass spectrometry to identify the total mass of a linker peptide attached to H287C AChE after releasing it by disulfide reduction.
- We also showed that we could obtain sequence information on MTS-linker-peptides with our ESI ion trap mass spectrometer and MS/MS analyses by collision activated decomposition (CAD). Fragmentation of the linker peptide corresponding to compound III (Table 1) resulted in cyclic ring opening and release of fragments that provided information about the cyclic peptide sequence.

REPORTABLE OUTCOMES

BOOK CHAPTERS

Johnson, J.L., Cusack, B., Davies, M.P., Fauq, A. and Rosenberry, T.L. Substrate activation with a cationic acetanilide substrate in human acetylcholinesterase. In *Cholinesterases in the Second Millennium: Biomolecular Pathological Aspects*. p. 213-218, N.C Inestrosa and E.O. Campos (eds) 2004, Diseno e Impresiones J&J Ltda., Chile

Cusack, B., Johnson, J.L., Hughes, T.F., McCullough, E.H., Fauq, A., Romanovskis, P.V., Spatola, A.F. and Rosenberry, T.L. Tethering of ligands near the active site of acetylcholinesterase mutant H287C: Progress on a new strategy for protection against organophosphate inactivation. In *Cholinesterases in the Second Millennium: Biomolecular Pathological Aspects*. p. 259-264, N.C Inestrosa and E.O. Campos (eds) 2004, Diseno e Impresiones J&J Ltda., Chile

PAPERS

Romanovskis, P., Rosenberry, T.L., Cusack, B., Spatola, A.F. Methanethiosulfonyl-derivatives of linear and cyclic peptides as novel reagents to study the topology of the active center gorge of acetylcholinesterase. *Biopolymers*. 71:399-399, (2003).

Johnson, J.L., Cusack, B., Hughes, T. F., McCullough, E.H., Fauq, A., Romanovskis, P., Spatola, A.F., and Rosenberry, T.L. Inhibitors tethered near the acetylcholinesterase active site serve as molecular rulers of the peripheral and acylation sites. *Journal of Biological Chemistry*. 278(40):38948-3894855, (2003).

Johnson, J.L., Cusack, B., Davies, M.P., Fauq, A. and Rosenberry, T.L. Unmasking tandem site interaction in human acetylcholinesterase. Substrate activation with a cationic acetanilide substrate. *Biochemistry*. 42:50438-50452, (2003).

ABSTRACTS

Johnson, J.L., Cusack, B., Hughes, T. F., McCullough, E.H., Fauq, A., Romanovskis, P., Spatola, A.F., and Rosenberry, T.L. Inhibitors tethered near the acetylcholinesterase active site serve as molecular rulers of the peripheral and acylation sites. *American Chemical Society*, September, 2003.

CONCLUSIONS

During the current grant year we have advanced our strategy of tethering cyclic peptides to the Cys residue in H287C AChE in a number of ways. Several synthesis schemes for linker groups that attach the MTS substituent to the cyclic peptide have been compared and evaluated for ease of synthesis, optimum length and solubility. Refinements now include insertion of a hydrophilic group (8-amino-3,6-dioxaoctanoic acid or mini-PEG) to increase solubility and hydrophilicity and optional substitution of the N-beta amino group of DAP (2,3-diaminopropionic acid) for the N-epsilon-amino group of Lys to vary the length of the linker. Some 15 new MTS-linker-peptides have been investigated (Tables 1 and 2). We focused on a group of compounds based on the cyclic peptide c(Arg-3AMB-Leu-Phe-X-Gln), where AMB was 3-(aminomethyl)benzoic acid and X was either Lys, D-Lys or DAP (Table 1). These cyclic peptides were selected because they showed some affinity for the P-site as reversible AChE inhibitors. AChE modification with only 2 of the MTS-linker-peptides (IV and VII in Table I) resulted in any enzyme release from the affinity resin in the salt wash, our selection criterion for peptides that P-site blockade. These results in general indicated that we need to increase the diversity of cyclic peptide structures that we are surveying, and we plan to pursue this goal with combinatorial syntheses and mass spectrometry identifications as outlined below. Our results also raised some concern that we were not achieving efficient modification of C287 with our MTS-linker-peptides, so we developed an assay with the neurotoxin fasciculin (Fas) to monitor this attachment. The assay measures k_{on} rate constants for Fas binding to the P-site, and we demonstrated that a reduction of k_{on} in this assay can confirm the attachment of linker peptide to H287C AChE even when the peptide fails to alter acridinium resin binding or enzyme activity.

We have also explored the use of mass spectrometry as a powerful tool to identify candidate cyclic peptides from initial combinatorial sets. Last year our Department of Neuroscience at Mayo Clinic Jacksonville purchased a ThermoFinnigan DecaXP Plus ESI ion trap mass spectrometer with liquid chromatography interface to support three projects, one of which was this project. In addition, the department also has an older Applied Biosystems, Voyager-DERP MALDI-TOF mass spectrometer that we have found useful. Bernadette Cusack, a senior research analyst in our laboratory, has spent much of the past year obtaining training and developing techniques in mass spectrometry, and we have gained perspective on the most effective ways to apply these techniques to our project. We demonstrated that we could apply MALDI TOF mass spectrometry to identify the total mass of a linker peptide attached to H287C AChE after releasing it by disulfide reduction. We also showed that we could obtain sequence information on MTS-linker-peptides with our ESI ion trap mass spectrometer and MS/MS analyses by collision activated decomposition (CAD). For example, fragmentation of the linker peptide corresponding to compound III (Table 1) resulted in cyclic ring opening at three peptide bond sites and release of four 3- to 5-residue fragments. These fragments provided information about the cyclic peptide sequence. We concluded from these studies that we could assign cyclic peptide structures selected from combinatorial peptide mixtures based on the combination of parent ion masses and specific sequence information from MS/MS analyses with CAD.

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